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TRF1 and TRF2 Differentially Modulate Rad51-Mediated Telomeric and Nontelomeric Displacement Loop Formation *in Vitro*

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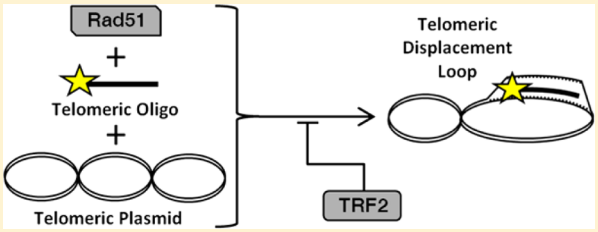
Supporting Information

ABSTRACT: A growing body of literature suggests that the homologous recombination/repair (HR) pathway cooperates with components of the shelterin complex to promote both telomere maintenance and nontelomeric HR. This may be due to the ability of both HR and shelterin proteins to promote strand invasion, wherein a single-stranded DNA (ssDNA) substrate base pairs with a homologous double-stranded DNA (dsDNA) template displacing a loop of ssDNA (D-loop). Rad51 recombinase catalyzes D-loop formation during HR, and telomere repeat binding factor 2 (TRF2) catalyzes the formation of a telomeric D-loop that stabilizes a looped structure in telomeric DNA (t-loop) that may facilitate telomere protection. We have characterized this functional interaction *in vitro* using a fluorescent D-loop assay measuring the incorporation of Cy3-labeled 90-nucleotide telomeric and nontelomeric substrates into telomeric and nontelomeric plasmid templates. We report that preincubation of a telomeric template with TRF2 inhibits the ability of Rad51 to promote telomeric D-loop formation upon preincubation with a telomeric substrate. This suggests Rad51 does not facilitate t-loop formation and suggests a mechanism whereby TRF2 can inhibit HR at telomeres. We also report a TRF2 mutant lacking the dsDNA binding domain promotes Rad51-mediated nontelomeric D-loop formation, possibly explaining how TRF2 promotes nontelomeric HR. Finally, we report telomere repeat binding factor 1 (TRF1) promotes Rad51-mediated telomeric D-loop formation, which may facilitate HR-mediated replication fork restart and explain why TRF1 is required for efficient telomere replication.



Mammalian telomeres consist of 5−15 kilobase pairs (kbp) of TTAGGG repeats that terminate in a 50−500-nucleotide (nt) single-stranded DNA (ssDNA) 3′ tail. The telomere repeats and the single-stranded−double-stranded DNA (ss−dsDNA) junction provide a binding site for telomere-specific proteins that shelter telomeres from being recognized as DNA damage. While these shelterin proteins may directly inhibit DNA damage signaling,[1,2] the presence of a DNA loop at the end of the telomeres (t-loop) may also mediate telomere protection. One shelterin component, telomere repeat binding factor 2 (TRF2), is required for t-loop formation *in vivo*[3] and can promote t-loop formation *in vitro*[4] by facilitating a strand invasion reaction between the ssDNA tail and upstream dsDNA in a telomere. However, telomere protection also requires components of the homologous recombination/repair (HR) pathway, which may facilitate telomere replication or promote t-loop formation.

In vitro, telomeric replication forks are prone to slipping,[5] and replication of telomeric DNA is inefficient[6] and prone to defects consistent with fork stalling.[7] *In vivo* fork stalling can be mitigated by proteins involved in the HR pathway.[8] Accordingly, replication of telomeric DNA *in vivo* is sensitive to disruption of that pathway. The BRCA2 tumor suppressor recruits the Rad51 recombinase to telomeres during replication, and disrupting the expression of either of these proteins results in telomere shortening and fragility. These phenotypes are attenuated in cells possessing short telomeres and are exacerbated by chemical inhibition of DNA replication.[9] As such, it is likely that these defects are due in part to a telomere replication defect.

Disrupting the HR pathway in nondividing cells results in aberrant telomere repair. Therefore, it is likely that the HR pathway also contributes to telomere protection in a replication-independent manner,[9] possibly by promoting t-loop formation. Concordantly, both TRF2 and Rad51 are required for cell extracts to promote telomeric D-loop formation,[10] a requisite step in t-loop formation. Interestingly, this relationship appears to be bidirectional. Overexpression of TRF2 promotes HR *in vivo*, while TRF2 knockdown inhibits HR *in vivo*.[11] While these observations suggest that TRF2 and HR cooperate functionally *in vivo*, this hypothesis contradicts these proteins' established *in vitro* activities. TRF2 induces positive supercoiling within telomeric dsDNA upon binding,[12] but Rad51 most efficiently promotes D-loop formation when acting upon negatively supercoiled dsDNA templates.[13]

To investigate functional interactions between shelterin proteins and the HR pathway, we undertook an *in vitro* characterization

Received: May 22, 2014
Revised: July 11, 2014
Published: August 12, 2014

   dx.doi.org/10.1021/bi5006249 | *Biochemistry* 2014, 53, 5485−5495

of the combined activities of purified proteins from these pathways. While the use of purified proteins permits an examination of their isolated functional interactions *in vitro*, such interactions may be affected by other proteins *in vivo*. The absence of such other proteins likely explains why the results of our assay contradict previous cell extract-based characterizations.[10] We report that TRF2 inhibits the ability of Rad51 to promote telomeric D-loop formation, suggesting that Rad51 does not promote t-loop formation and elucidating a novel mechanism by which TRF2 inhibits aberrant DNA repair at the telomeres. In contrast, we report that TRF1 promotes Rad51-mediated telomeric D-loop formation, possibly explaining why TRF1 is required for efficient telomere replication. Finally, we report that a TRF2 mutant lacking the dsDNA binding domain was able to promote Rad51-mediated D-loop formation, suggesting that one or more TRF2 domains can positively modulate Rad51 activity and possibly explaining how TRF2 can facilitate HR.

EXPERIMENTAL PROCEDURES

DNA Substrates, Templates, and Competitors. A pBluescript-derived plasmid containing a 103 bp telomeric tract [pBB, $(TTAGGG)_{17}T$] was generated by conventional cloning via insertion of the BsmBI/BbsI fragment of pRST15[4] into BsmBI-cut pRST15. A pBluescript-derived plasmid containing a nontelomeric insert (pGL GAP) was generated as previously described.[14] All plasmids were cultured in DH10B *Escherichia coli* and purified using Qiagen Maxiprep kits. High-performance liquid chromatography-purified 5′ Cy3-labeled G-rich telomeric 90-mer oligonucleotide [T90, $[Cy3](GGTTAG)_{15}$], D1 oligonucleotide ([Cy3]AAATCAATCTAAAGTATATATGAGTAAACTTGGTCTGACAGTTACCAATGCTTAATCAGTGAGGCACCTATCTCAGCGATCTGTCTATTT), and T3 promoter primer ([Cy3]ATTAACCCTCACTAAAGGA) and HPSF-purified unlabeled T7 promoter primer (TAATACGACTCACTATAGGG) were ordered from Eurofins MWG Operon. A 255 bp Cy3-labeled PCR product was amplified from pBB using the 5′ Cy3-labeled T3 and unlabeled T7 promoter primers and Q5 High Fidelity Polymerase (New England BioLabs) following the manufacturer's instructions and purified using a DNA Clean & Concentrator-25 column (Zymo Research).

Proteins. Untagged Rad51 was expressed and purified as previously described[15] from a pET-24-derived plasmid (EMD Millipore), which was generously provided by R. Fishel (The Ohio State University, Columbus, OH). N-Terminally hexahistidine-tagged TRF2, TRF2ΔB, TRF2ΔM, and TRF1 were purified from pTRC-HIS-derived plasmids (Invitrogen) adapted from vectors generously provided by the laboratory of E. Gilson[12] (University of Nice, Nice, France) or modified from vectors previously described.[16] All TRF2 cDNAs were modified to include the Ala434 codon that is absent in HeLa-derived TRF2 clones.[17] Briefly, a pTRC-HIS plasmid was transformed into BL21(DE3)PlysS *E. coli* and serially passaged to inoculate 1 L of Terrific Broth (Sigma-Aldrich) containing 50 μg/mL ampicillin. The culture was grown to an OD of 0.6 at 595 nm, and protein expression was then induced via addition of 1 mM isopropyl β-D-1-thiogalactopyranoside (Promega) for 4 h at 37 °C. The cells were then recovered via centrifugation, washed with phosphate-buffered saline, resuspended in 100 mL of buffer containing 20 mM HEPES (pH 7.5), 300 mM NaCl, 10% glycerol, 0.5 mM DTT, and 50 mM imidazole supplemented with protease inhibitors (Roche), and then flash-frozen in liquid nitrogen. The cells were then thawed and lysed via sonication following addition of 1 mg/mL egg white lysozyme and 20 μL of RQ1 DNase (Promega) and 20 μL of RNase A (Sigma). The crude lysate was then centrifuged in an SW-41 Ti rotor at 41000 rpm for 1.5 h. The supernatant was collected and serially purified over 1 mL HisTrap HP, HiTrap Heparin HP, and HiTrap Q FF columns using an ÄKTApurifier FPLC (GE Bioscience). Rad51, TRF2, TRF2ΔB, and TRF2ΔM protein were recovered in 20 mM HEPES (pH 7.5), 150 mM NaCl, 10% glycerol, and 0.5 mM DTT, while TRF1 was recovered in 20 mM HEPES (pH 7.5), 300 mM NaCl, 10% glycerol, and 0.5 mM DTT. These proteins were aliquoted, flash-frozen with liquid nitrogen, and stored at −80 °C until they were used. The protein concentration was determined using a Bio-Rad Protein Assay calibrated against a Bovine Gamma Globulin standard set (Bio-Rad). For all proteins, homogeneity was assessed as being >90% by Coomassie staining of sodium dodecyl sulfate−polyacrylamide gel electrophoresis gels. Immediately prior to being used in experiments, TRF2, TRF2ΔB, TRF2ΔM, and TRF1 were diluted to a final concentration of 4.25 μM in buffer containing 19 mM HEPES-KOH, 203.8 mM NaCl, 1 mM CaCl, 1 mM ATP, 7% glycerol, and 0.7 mM DTT. All protein concentrations are reported as monomeric protein. Rad51 was purified to a concentration of 27.5 μM and was used undiluted in all experiments. Fraction V bovine serum albumin (Fisher) was diluted to 10 mg/mL in 20 mM potassium phosphate (pH 7.0), 50 mM NaCl, 5% glycerol, and 0.1 mM EDTA.

Displacement Loop Assay. For the displacement loop assay, the 5′ Cy3-labeled telomeric 90-mer [2.4 μM in nucleotides (nt), 26.67 nM oligo] was incubated with no protein or 1000−1500 nM Rad51 at 37 °C for 10 min in a reaction buffer containing 5 mM HEPES-KOH (pH 7.5), 1 mM CaCl, 1 mM ATP, 0.8 mM DTT, and 100 μg/mL BSA. Simultaneously, the pBB plasmid, 35 μM in base pairs, or 10 nM plasmid was incubated with no protein or 100−500 nM TRF2, TRF2ΔB, TRF2ΔM, or TRF1 at 37 °C for 10 min in reaction buffer and 100 μg/mL BSA. Equal volumes of these reaction mixtures were then combined to give final Rad51 concentrations of 0 or 500−750 nM and a final concentration of 0−250 nM TRF2, TRF2ΔB, TRF2ΔM, or TRF1. These reaction mixtures were incubated at 37 °C for 1 h and then deproteinized via addition of 0.5% sodium dodecyl sulfate and 1 mg/mL proteinase K (Ambion) and incubated at 37 °C for 15 min. Glycerol loading buffer (5% glycerol, 1.67 mM Tris, 0.17 mM EDTA, and 0.017% SDS) was then added to a concentration of 1×, and the samples were separated for 30 min in a small-format 1% $^1/_2$× TBE agarose gel at 100 V (6.67 V/cm) in a light-protected box in a 4 °C cold room. All figures are labeled with the final respective protein concentrations.

Electrophoretic Mobility Shift and Binding Competition Assay. To demonstrate binding via an electrophoretic mobility shift assay, Cy3-labeled PCR product (2.55 μM in bp, 10 nM product) was incubated with no protein or 100−500 nM TRF2, TRF2ΔB, TRF2ΔM, or TRF1 at 37 °C for 10 min in reaction buffer supplemented with 100 μg/mL BSA. To demonstrate binding specificity via a competition assay, an additional set of 500 nM reactions were performed in a buffer containing no competitor or between a 1:1 (2.55 μM in base pairs) and 200:1 (510 μM in base pairs) excess of pGL GAP and then incubated at 37 °C for 25 min. To demonstrate that the induced supershifts were protein-mediated, a 500 nM reaction mixture containing no competitor was incubated for 10 min and then deproteinized with SDS and proteinase K for 15 min.

dx.doi.org/10.1021/bi5006249 | *Biochemistry* 2014, 53, 5485−5495

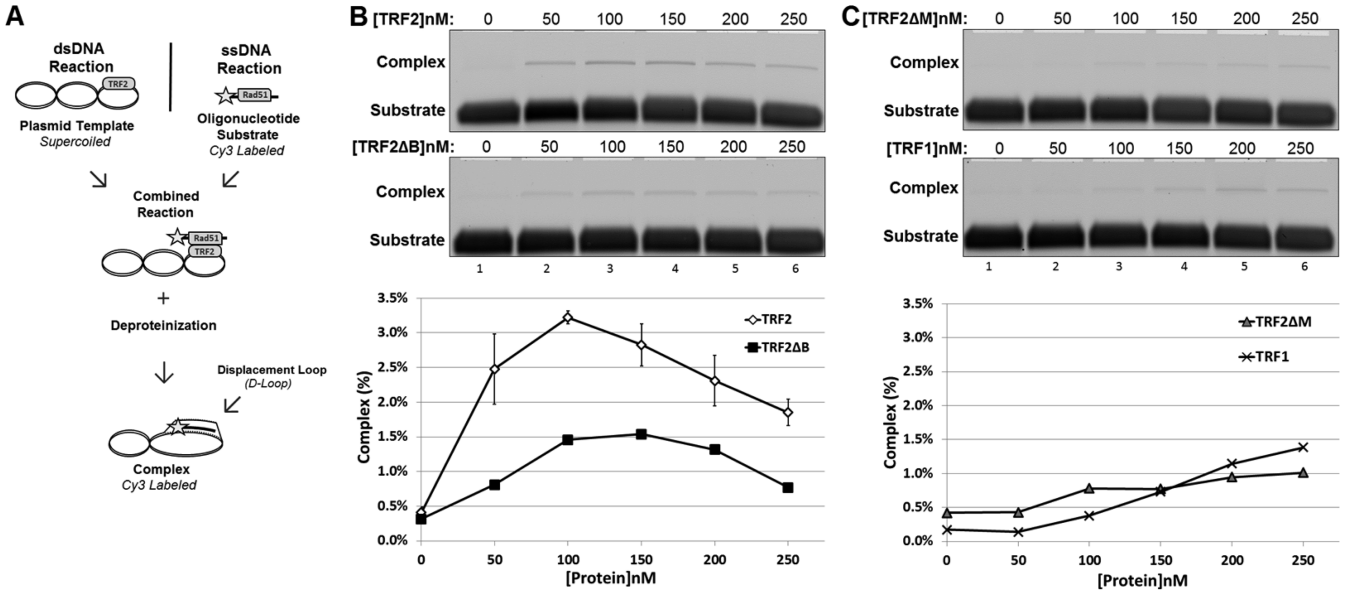


Figure 1. TRF-mediated telomeric D-loop formation. (A) Diagram of the D-loop assay. (B) TRF2 and TRF2ΔB promote telomeric D-loop formation with an activity peak when included at a final protein concentration between 100 nM (lane 3) and 150 nM (lane 4). (C) TRF2ΔM and TRF1 promote telomeric D-loop formation only at higher concentrations.

Glycerol loading buffer containing no SDS was then added to a concentration of 1×, and the samples were separated for 30 min in a small-format $^{1}/_{2}$× TBE agarose gel at 100 V (6.67 V/cm) in a light-protected box in a 4 °C cold room. All figures are labeled with the final respective protein concentrations.

Imaging. All Cy3-labeled gel products were imaged using a General Electric Typhoon 9400 Scanner equipped with a 532 nM green laser module and a 580 nM bypass filter. Gels were imaged with a photomultiplier setting of 600 and a pixel size of 100 μm. All gels were imaged with a +3 mm focal plane setting. The gel image intensity was then adjusted using ImageQuant (GE Life Sciences) and quantified using ImageJ (National Institutes of Health, Bethesda, MD).

RESULTS

A Fluorescent TRF2 and Rad51-Mediated Displacement Loop Assay. To investigate functional interactions between Rad51 and TRF2, we developed a fluorescent displacement loop (D-loop) assay (Figure 1A) adapted from previous TRF2 and Rad51 characterizations.[12,15] Untagged Rad51 and N-terminally hexahistidine-tagged TRF1, TRF2, and TRF2 mutant proteins lacking either the N-terminal basic domain of TRF2 (TRF2ΔB) or the C-terminal Myb domain of TRF2 (TRF2ΔM) were purified from *E. coli* to >90% homogeneity (Figure S1 of the Supporting Information). In this assay, co-incubation of a Cy3-labeled telomeric ssDNA substrate (T90) with a dsDNA telomeric plasmid template (pBB) in the absence of any proteins resulted in low to undetectable levels (<0.5%) of spontaneous D-loop formation (Figure 1B,C, lane 1). In contrast, preincubation of the substrate with purified Rad51 protein prior to its addition to the template promoted D-loop formation in a Rad51 concentration-dependent manner (Figure 2A,B). Likewise, preincubation of the template with full length TRF2 protein prior to its addition to the substrate could promote D-loop formation across a discrete range of TRF2 concentrations (Figure 1B). TRF2ΔB exhibited only 47% of the activity of full length TRF2 (Table 1), but this residual activity was similarly optimal across a narrow range of concentrations (Figure 1B). In contrast, TRF2ΔM and TRF1 exhibited only 31 and 27%, respectively, of the activity of full length TRF2 (Table 1) and were maximally active only at higher concentrations (Figure 1C).

Rad51-mediated D-loop formation was observed to be homology-driven. Rad51 could promote D-loop formation between telomeric substrates and templates, and nontelomeric substrates and templates, but not between a telomeric substrate and a nontelomeric template (Figure S2A,B of the Supporting Information). Under identical conditions, the extent of Rad51-mediated telomeric D-loop formation was 6.4-fold higher (Table 1) than the extent of nontelomeric D-loop formation (Figure S2 of the Supporting Information). This is consistent with previous characterizations showing that the activity of Rad51 is enhanced on repetitive and GT-rich substrates.[18,19] In contrast to Rad51, TRF2-mediated D-loop formation was observed to be critically dependent upon telomeric homology. TRF2 could promote D-loop formation only between telomeric substrates and templates (Figure S2C,D of the Supporting Information).

TRF2 Inhibits Rad51-Mediated Telomeric but Not Nontelomeric D-Loop Formation. To test for functional interactions between TRF2 and Rad51, D-loop assay reaction mixtures in which the template was preincubated with either a fixed concentration of TRF2 or no protein were prepared, while the substrate was preincubated with one of several concentrations of Rad51 or no protein prior to the combination of the substrate and template reaction mixtures. Preincubation of a telomeric template with TRF2 weakened the ability of Rad51 to promote D-loop formation between the template and a homologous telomeric substrate by 52 ± 5.1% (Table 1 and Figure 2A,B). In contrast, TRF2 did not significantly inhibit Rad51-mediated nontelomeric D-loop formation (Table 1 and Figure 2C,D). Taken together, these data suggested that TRF2 differentially modulates Rad51-mediated telomeric and nontelomeric D-loop formation.

Rad51-mediated D-loop formation is a multistep process initiated by binding of Rad51 to ssDNA to form a nucleoprotein

dx.doi.org/10.1021/bi5006249 | *Biochemistry* 2014, 53, 5485−5495

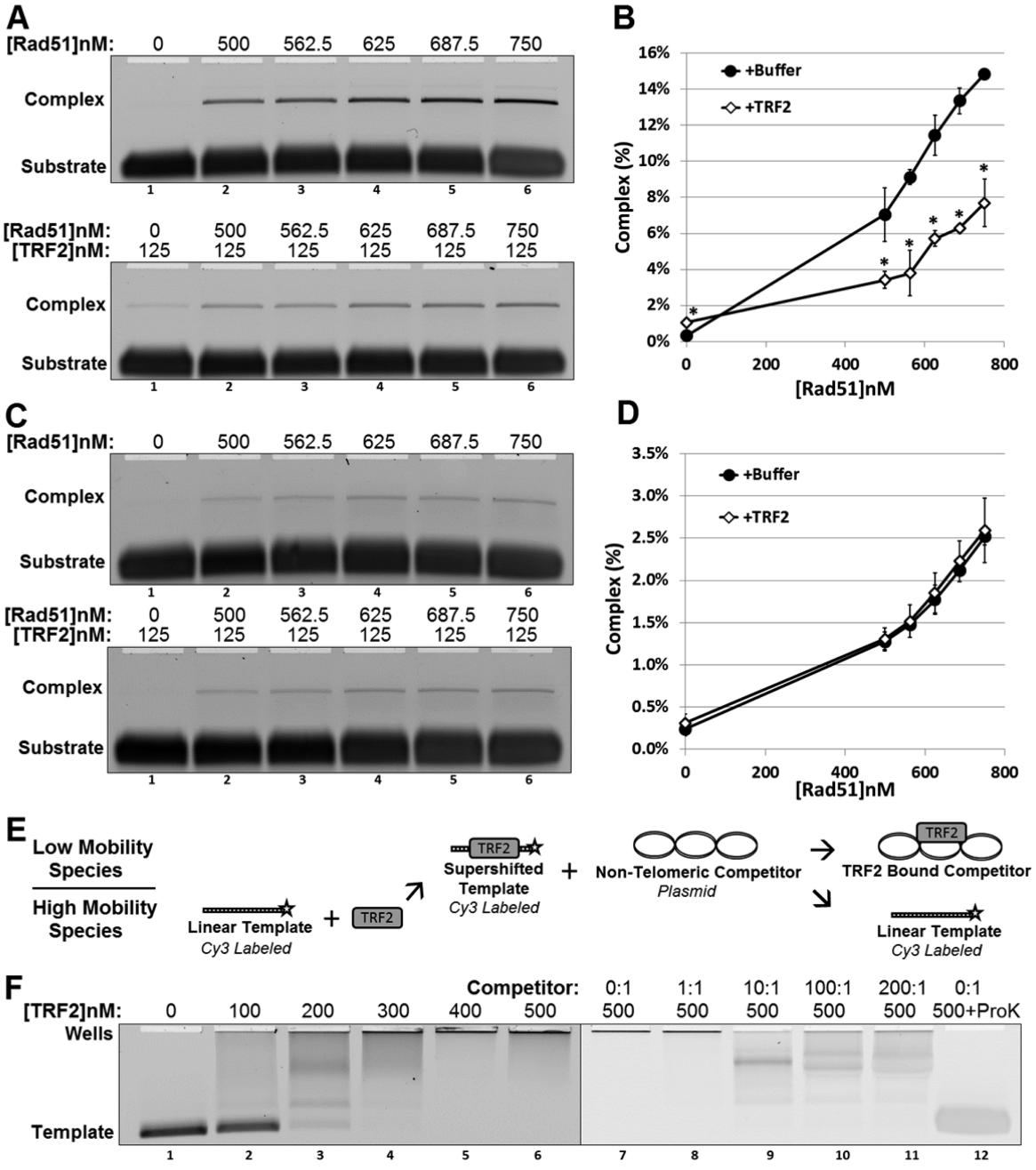


Figure 2. TRF2 inhibits Rad51-mediated telomeric but not nontelomeric D-loop formation. (A) Rad51 promotes telomeric D-loop formation in a concentration-dependent manner that is inhibited by TRF2. (B) Quantification of data in panel A. (C) Rad51 promotes nontelomeric D-loop formation in a concentration-dependent manner that is not affected by TRF2. (D) Quantification of the data depicted in panel C. (E) Diagram of the DNA binding and competition assay using a Cy3-labeled PCR product containing the 103 bp telomere tract from pBB. (F) TRF2 binding supershifts the template into the wells. This binding is specific, persists in the presence of high concentrations of nontelomeric competitor, and is protein-mediated. Error bars show the 95% confidence interval; asterisks denote the significant difference between +Buffer and +TRF2, via the paired sample t test $\alpha = 0.05$ from three independent experiments.

filament, which subsequently interrogates dsDNA for matching antisense sequence in a process known as "homology search". In this process, a Rad51-coated substrate initially forms a protein-mediated complex with a homologous template. Subsequently, Rad51 promotes D-loop formation between the substrate and template.[20] To determine what step or steps of this process might be inhibited by TRF2, we performed several order of addition experiments.

We observed that the ability of TRF2 to inhibit Rad51-mediated telomeric D-loop formation was dependent upon addition of TRF2 early in the D-loop reaction (Figure S3 of the Supporting Information). TRF2 could inhibit Rad51-mediated telomeric D-loop formation upon being preincubated with the telomeric template (T0) or upon being added to a combined reaction prior to D-loop formation (time zero + 10 min). However, TRF2 could not inhibit Rad51-mediated D-loop formation if it were added after D-loop formation had already occurred (time zero + 3 h). These observations suggested that TRF2 modulates Rad51-mediated D-loop formation via a passive mechanism, possibly by interfering with Rad51 filament

dx.doi.org/10.1021/bi5006249 | *Biochemistry* 2014, 53, 5485−5495

Table 1. Properties of TRF2, TRF2ΔB, TRF2ΔM, and TRF1[a]

protein	% of TRF2	% change from buffer		telomeric DNA binding, $C_{1/2}$ (nM)	telomeric binding specificity	migration in agarose gels
	TRF-induced telomeric D-loop formation (AUC at 0−250 nM)	Rad51-mediated telomeric D-loop formation (AUC at 500−750 nM)	Rad51-mediated nontelomeric D-loop formation (AUC at 500−750 nM)			
buffer	N/A	30.3 ± 0.4	4.7 ± 0.4	N/A	+	−
		0 ± 0.7%	0 ± 7.6%			
TRF2	6.0 ± 0.15	13.3 ± 0.8[b]	4.7 ± 0.0	111 ± 8	+	−
	100 ± 2.4%	−52 ± 5.1%[b]	+4 ± 2.2%			
TRF2ΔB	2.8	22.1 ± 0.7[b]	5.1 ± 0.7	257	+	−
	47%	−31 ± 5.5%[b]	+5 ± 7.2%			
TRF2ΔM	1.8	29.0 ± 1.4	9.6 ± 0.4[b]	319	−	+/−
	31%	+4 ± 4.0%	+112 ± 13.0%[b]			
TRF1	1.6	38.5 ± 1.9[b]	5.4 ± 0.9	152	+	+
	27%	+25 ± 1.0%[b]	+9 ± 5.2%			

[a]Buffer data are averaged. Proteins are statistically compared against matched buffer controls. TRF-induced D-loop formation calculated as the area under the curve (AUC, % complex × [TRF] in nanomolar) from Figure 1. Rad51-mediated D-loop formation calculated as AUC (% complex × [Rad51] in nanomolar) from Figures 2−5. AUC calculation examples in Figure S5 of the Supporting Information. $C_{1/2}$ represents the concentration of TRF protein required to supershift 50% of the template in EMSAs. Errors shown are 95% confidence intervals from three independent experiments. [b]Significant difference ($p < 0.05$) from buffer via a two-tailed paired sample t test.

formation, inhibiting homology search, or preventing subsequent D-loop formation.

We also observed that TRF2 could inhibit telomeric D-loop formation regardless of whether TRF2 was preincubated with the telomeric template or with the Rad51-coated substrate (Figure S4 of the Supporting Information). However, the degree of this inhibition was reduced when TRF2 was incubated with the substrate compared with when it was incubated with the template. This suggests that TRF2 does not inhibit Rad51 at the level of filament formation. Instead, it appears that the ability of TRF2 to inhibit Rad51-mediated telomeric D-loop formation is dependent upon the ability of TRF2 to bind to or modify the template.

To investigate whether the DNA binding activities of TRF2 mediate its ability to inhibit Rad51-mediated telomeric D-loop formation, we characterized the binding affinity and specificity of TRF2 using an electrophoretic mobility shift assay (EMSA) and a binding competition assay (Figure 2E). Incubating a Cy3-labeled PCR product containing a 103 bp telomere track with increasing concentrations of TRF2 resulted in a supershift of that template, consistent with stable TRF2 binding (Figure 2F). The binding of TRF2 to the template was observed to be specific and persisted even in the presence of high concentrations of nontelomeric competitor (Figure 2F, lanes 8−11). Nearly all low-mobility species generated by TRF2 binding became trapped in the wells. This supershift was protein-mediated and could be disrupted by incubation with SDS and proteinase K (Figure 2F, lane 12).

To further investigate the possible mechanism by which TRF2 may inhibit Rad51-mediated telomeric D-loop formation, we characterized the binding activity and telomeric and nontelomeric Rad51-modulating activities of a variety of TRF2 mutant proteins and TRF1, a close homologue of TRF2.

TRF2ΔM Promotes Rad51-Mediated Telomeric but Not Nontelomeric D-Loop Formation. The dsDNA binding activity of TRF2 is primarily directed by its C-terminal Myb domain. Deletion of this Myb domain reduces telomeric dsDNA binding affinity by a factor of 2.9 (Table 1), eliminates telomeric binding specificity, and grossly alters DNA binding properties compared to those of full length TRF2 (Figure 3E).[12] Interestingly and in contrast to TRF2, TRF2ΔM was found to promote Rad51-mediated nontelomeric D-loop formation by 112 ± 13.0% (Table 1 and Figure 3C,D), despite the impaired DNA binding. TRF2ΔM was observed to promote Rad51-mediated telomeric D-loop formation, albeit only at the lowest concentration tested (Table 1 and Figure 3A,B). However, TRF2ΔM did not promote Rad51-mediated telomeric D-loop formation across the entire concentration range tested, possibly because of the already high efficiency of the reactions. Taken together, these observations suggest that the Myb domain of TRF2 both contributes to the ability of TRF2 to inhibit Rad51-mediated telomeric D-loop formation and suppresses the ability of TRF2 to promote Rad51-mediated nontelomeric D-loop formation.

TRF2ΔB Inhibits Telomeric but Not Nontelomeric Rad51-Mediated D-Loop Formation. In addition to its Myb domain, TRF2 possesses an N-terminal domain rich in basic residues that has been implicated in directing the binding of TRF2 to ss−dsDNA junctions and unusual DNA structures.[4,21] This domain also promotes the annealing and migration of DNA joints in a manner not unlike that required during D-loop formation.[22] To investigate whether the basic domain of TRF2 contributes to the ability of TRF2 to inhibit Rad51-mediated

dx.doi.org/10.1021/bi5006249 | Biochemistry 2014, 53, 5485−5495

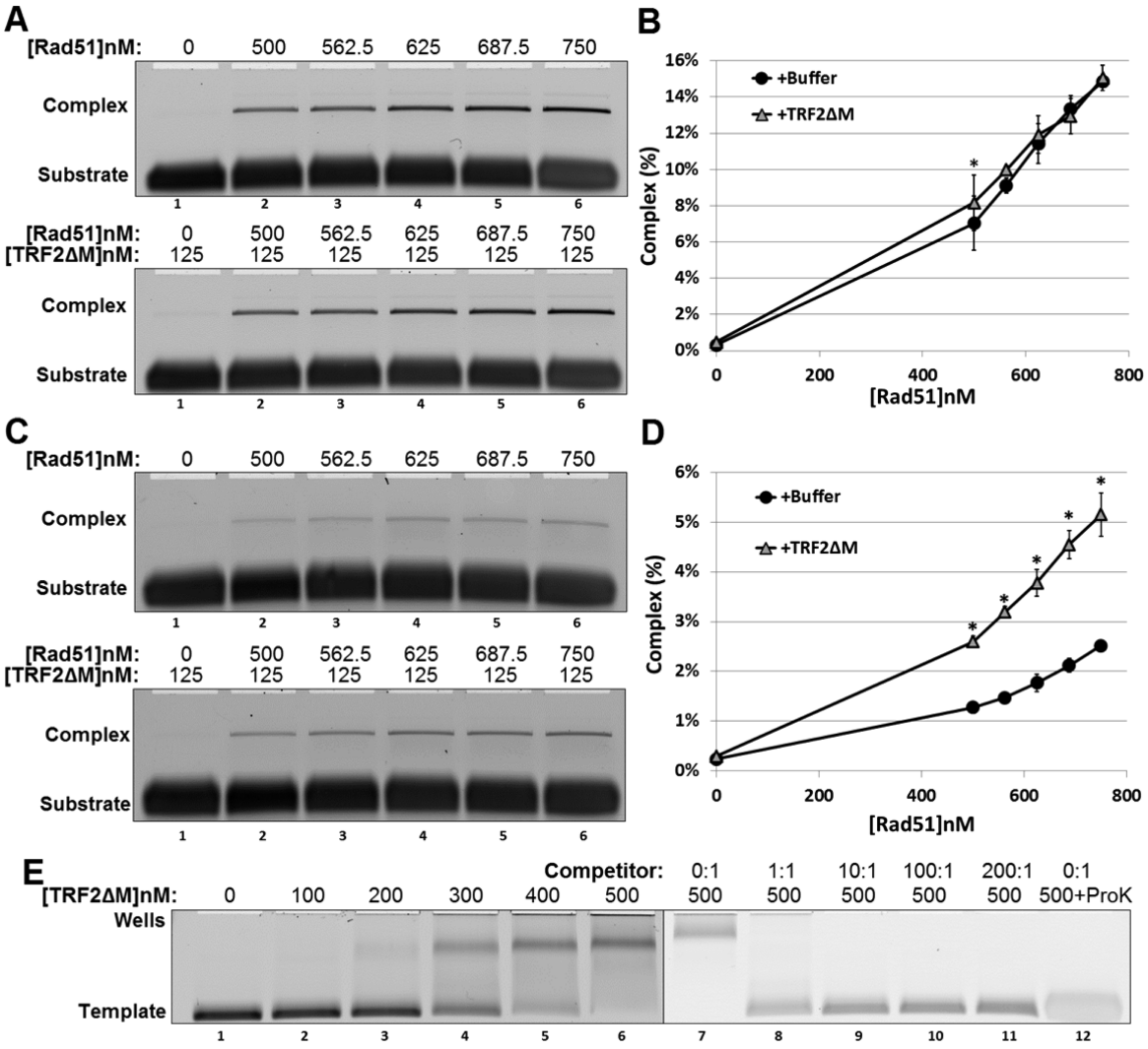


Figure 3. TRF2ΔM promotes Rad51-mediated nontelomeric but not telomeric D-loop formation. (A) Rad51 promotes telomeric D-loop formation in a concentration-dependent manner that is not affected by TRF2ΔM. (B) Quantification of the data depicted in panel A. (C) Rad51 promotes nontelomeric D-loop formation in a concentration-dependent manner that is promoted by TRF2ΔM. (D) Quantification of the data depicted in panel C. (E) TRF2ΔM binding supershifts the template into a lower-mobility species and into the wells. This binding is nonspecific and is disrupted by low concentrations of nontelomeric competitor and is protein-mediated. Error bars show the 95% confidence interval; asterisks denote significant differences between +Buffer and +TRF2ΔM, via the paired sample *t* test $\alpha = 0.05$ from three independent experiments.

telomeric D-loop formation or the ability of TRF2ΔM to promote Rad51-mediated nontelomeric D-loop formation, we characterized the DNA binding affinity and specificity and Rad51-modulating activity of a TRF2 mutant protein lacking the basic domain of TRF2 (TRF2ΔB).

Like TRF2 and in contrast to TRF2ΔM, TRF2ΔB was found to inhibit Rad51-mediated telomeric D-loop formation by 31 ± 5.5% (Table 1 and Figure 4A,B), suggesting that the joint binding activity of TRF2 is not required for TRF2 to inhibit Rad51-mediated telomeric D-loop formation. In contrast, TRF2ΔB was not observed to affect Rad51-mediated nontelomeric D-loop formation (Table 1 and Figure 4C,D). Deletion of the basic domain resulted in an approximately 2.3-fold reduction in template binding affinity (Table 1) but did not reduce binding specificity (Figure 4E, lanes 8−11) compared to that of full length TRF2. Like TRF2, TRF2ΔB binding resulted in the template becoming trapped in the wells.

TRF1 Promotes Rad51-Mediated Telomeric but Not Nontelomeric D-Loop Formation. Our observation that TRF2 and TRF2ΔB but not TRF2ΔM could inhibit Rad51-mediated telomeric D-loop formation suggested that this inhibition could simply be due to Myb domain-directed dsDNA binding. To test this hypothesis, we characterized the DNA binding and Rad51 modulating activity of TRF1, a TRF2 homologue with a highly similar Myb domain (Figure S1A of the Supporting Information).[23] Interestingly and in contrast to TRF2, TRF1 was found to promote Rad51-mediated telomeric D-loop formation by 25 ± 1.0% (Table 1 and Figure 5A,B), suggesting that the ability of TRF2 to inhibit this process is not simply due to Myb domain binding. In contrast, TRF1 was found not to affect Rad51-mediated nontelomeric D-loop formation (Figure 5C,D). Comparisons between TRF1 and TRF2 must be made with caution, as despite the comparable DNA binding affinities and telomeric sequence specificities (Table 1) their binding behavior is otherwise grossly different upon examination via an EMSA. Whereas TRF2 binding shifts a telomeric template into the wells (Figure 2F), TRF1 binding shifts the species into increasingly larger complexes as the TRF1 concentration is increased (Figure 5E). This behavior is perhaps consistent with previous observations that while TRF2 binds to

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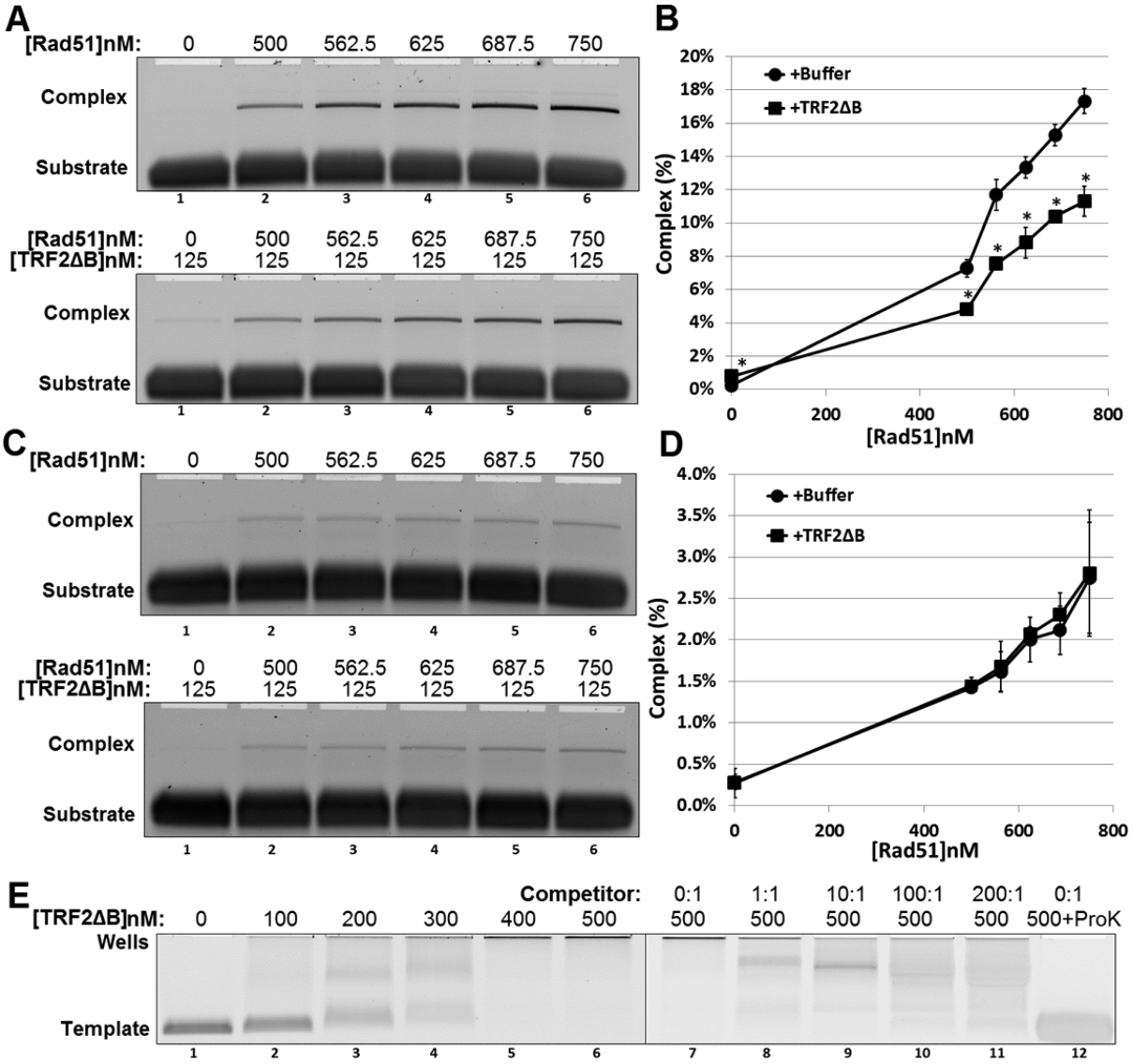

Figure 4. TRF2ΔB promotes Rad51-mediated telomeric but not nontelomeric D-loop formation. (A) Rad51 promotes telomeric D-loop formation in a concentration-dependent manner that is promoted by TRF2ΔB. (B) Quantification of the data depicted in panel A. (C) Rad51 promotes nontelomeric D-loop formation in a concentration-dependent manner that is not affected by TRF2ΔB. (D) Quantification of the data depicted in panel C. (E) TRF2ΔB binding supershifts the template into the wells. This binding is specific, persists in the presence of high concentrations of nontelomeric competitor, and is protein-mediated. Error bars show the 95% confidence interval; asterisks denote the significant difference between +Buffer and +TRF2ΔB, via the paired sample *t* test $\alpha = 0.05$ from three independent experiments.

telomeric dsDNA as a large oligomeric structure, TRF1 binds as a smaller complex.[24,25] Likewise, this property may be consistent with observations that TRF2 can promote the formation of unusual DNA structures and induce topological changes within telomeric DNA to a greater degree than TRF1.[12,26]

DISCUSSION

The results of this study suggest a model in which TRF1 and TRF2 differentially regulate Rad51-mediated telomeric and nontelomeric D-loop formation. This may promote efficient telomeric DNA replication and nontelomeric HR while inhibiting aberrant HR at the telomeres. TRF1 promotes Rad51-mediated telomeric D-loop formation, which may facilitate replication fork restart and explain why TRF1 is required for efficient telomere replication *in vivo*. In contrast, TRF2 inhibits Rad51-mediated telomeric D-loop formation, demonstrating a novel mechanism by which TRF2 may inhibit telomeric DNA repair. Finally, TRF2ΔM promotes Rad51-mediated D-loop formation, providing insight into how TRF2 may contribute to HR. Our findings are generally in good agreement with previous *in vitro* characterizations, and what contradictions exist are likely due to methodological differences.

Our D-loop assay is adapted from previous assays used to characterize purified Rad51 and TRF2.[12,15] The use of purified proteins permits direct interrogation of their functional interaction under controlled conditions across wide concentration ranges using defined orders of addition. However, data from *in vitro* characterizations should be compared with data from *in vivo* and genetic characterizations with caution. The activities of TRF1, TRF2, and Rad51 are modulated *in vivo* by other shelterin and HR proteins, which are absent in our assays. Additionally, the templates and substrates used in our assay differ from their *in vivo* analogues. Telomeres contain ss–dsDNA junctions, which were absent from the substrates used

dx.doi.org/10.1021/bi5006249 | *Biochemistry* 2014, 53, 5485–5495

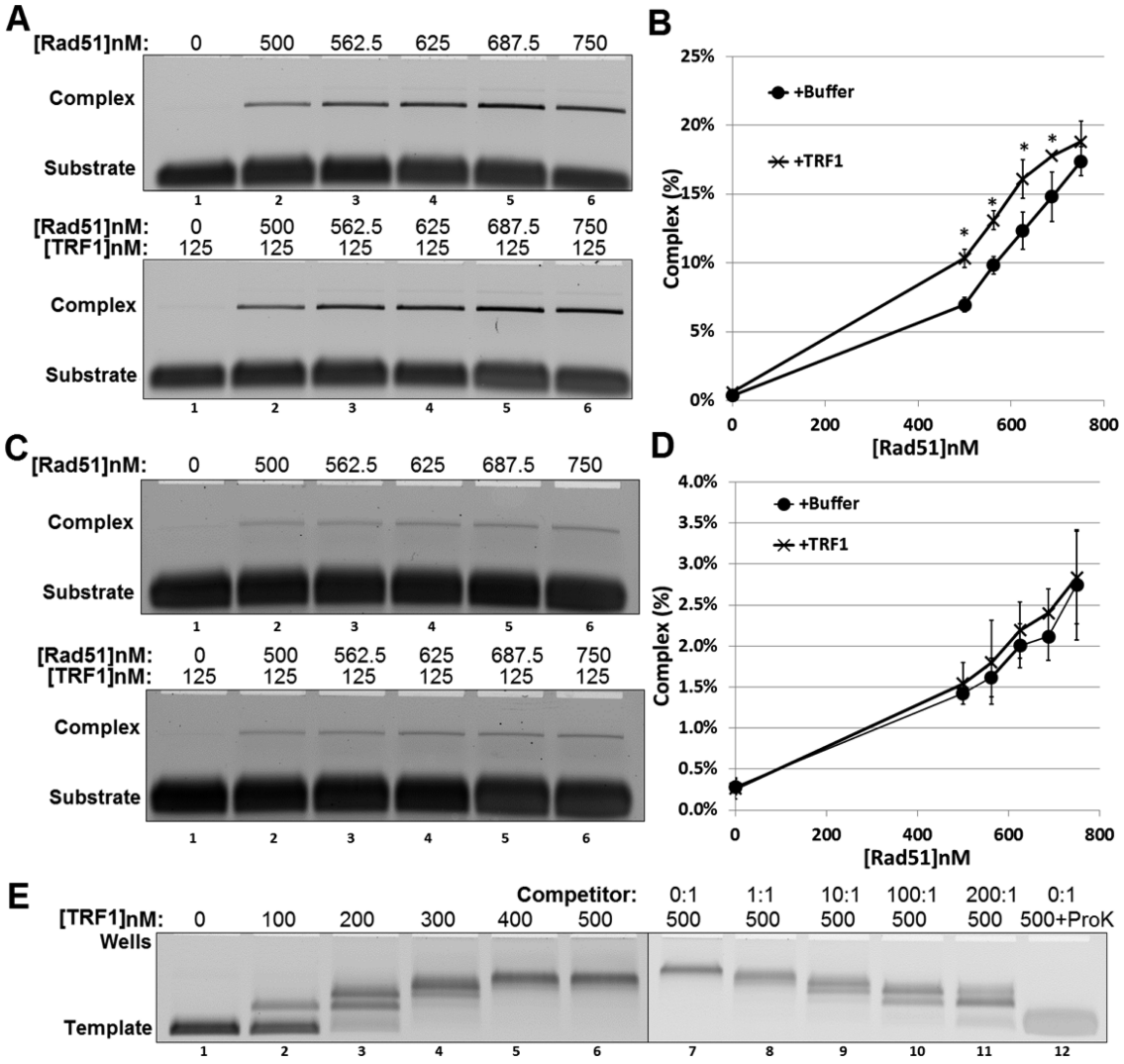

Figure 5. TRF1 promotes Rad51-mediated telomeric but not nontelomeric D-loop formation. (A) Rad51 promotes telomeric D-loop formation in a concentration-dependent manner that is promoted by TRF1. (B) Quantification of the data depicted in panel A. (C) Rad51 promotes nontelomeric D-loop formation in a concentration-dependent manner that is not affected by TRF1. (D) Quantification of the data depicted in panel C. (E) TRF1 binding supershifts the template into several low-mobility species. This binding is specific, persists in the presence of high concentrations of nontelomeric competitor, and is protein-mediated. Error bars show the 95% confidence interval; asterisks denote the significant difference between +Buffer and +TRF1, via the paired sample *t* test $\alpha = 0.05$ from three independent experiments.

in this work. Furthermore, telomeres are several kilobase pairs in length, whereas our telomeric template possessed only a 103 bp telomeric tract to permit comparison of telomeric and nontelomeric D-loop formation. Finally, while the topology of telomeric DNA *in vivo* is unknown, the templates used in our assay were negatively supercoiled.

While we observed that TRF2 inhibits Rad51, it has previously been reported that TRF2 and Rad51 cooperate functionally. Immunodepletion of TRF2 or Rad51 from nuclear extracts ablates the ability of those extracts to promote telomeric D-loop formation,[10] and supplementation of the immunodepleted extracts with Rad51 or TRF2 restores telomeric D-loop formation.[10,27] However, these nuclear extract data appear to be at odds with recent findings that expression of TRF2 inhibits HR-mediated telomeric plasmid integration and excision in yeast strains with humanized telomeres.[28] These discrepancies may reflect the presence of different factors in mammalian and yeast nuclei, which were absent from our *in vitro* assays. Moreover, discrepancies between the D-loop assays may stem from differences in incubation times, order of addition, and the structure, concentrations, and stoichiometry of the template and substrate.

The fact that TRF2 and TRF2ΔB but not TRF2ΔM inhibit Rad51-mediated telomeric D-loop formation may seem to suggest that this inhibition is due to Myb domain-directed telomeric dsDNA binding. However, TRF1 promotes Rad51-mediated telomeric D-loop formation despite having a homologous Myb domain. These data suggest that Myb domain binding is necessary but not sufficient for inhibition of Rad51-mediated D-loop formation. The Myb domain may be required to recruit additional domains that inhibit Rad51 via other processes. Differential modulation of Rad51-mediated D-loop formation by TRF1 and TRF2 may stem from differences in their oligomerization or their ability to promote supercoiling within telomeric dsDNA. Unfortunately, mechanisms of TRF1 and TRF2 oligomerization and supercoiling induction are not fully understood and are mediated by overlapping sets of protein domains.

dx.doi.org/10.1021/bi5006249 | *Biochemistry* 2014, 53, 5485−5495

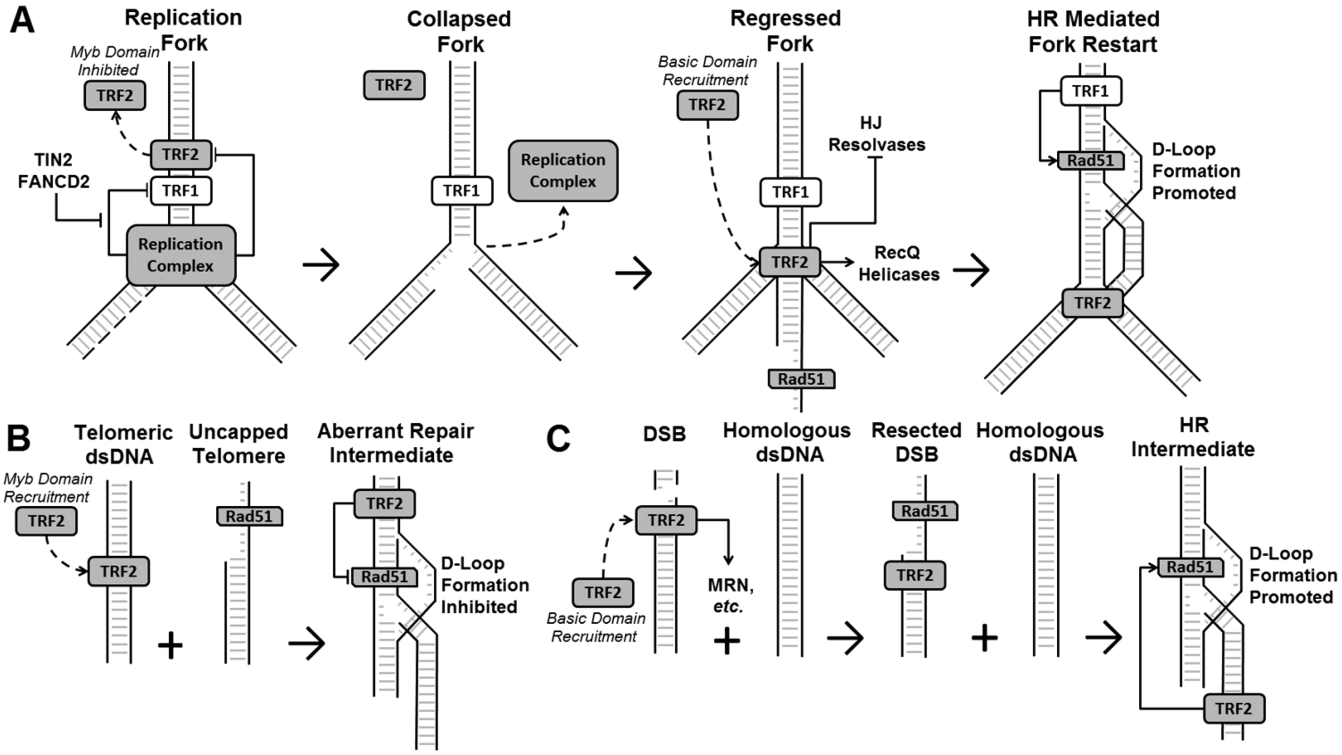

Figure 6. TRF1 and TRF2 differentially modulate Rad51-mediated telomeric and nontelomeric D-loop formation. (A) Post-translational modifications may deplete TRF2 but not TRF1 from telomeric DNA near a replication fork, possibly by inhibiting TRF2 Myb domain binding. Following fork collapse, basic domain-directed TRF2 binding can protect regressed forks from cleavage and recruit factors that promote fork migration. Finally, TRF1 can promote D-loop formation away from the fork and thereby promote HR-mediated fork restart. (B) TRF2 inhibits Rad51-mediated telomeric D-loop formation, which may prevent aberrant repair processes at the telomeres. (C) TRF2 is recruited to DSBs, where it may promote recruitment of enzymes that promote end resection. Afterward, the basic domain of TRF2 may promote Rad51-mediated D-loop formation and thereby promote HR.

TRF2 promotes positive supercoiling within telomeric dsDNA upon binding, whereas TRF1 does not.[12] This is of note, as Rad51-mediated D-loop formation is most efficient when acting on negatively supercoiled templates.[13] In contrast to the N-terminal basic domain of TRF2, the N-terminus of TRF1 is rich in acidic residues and appears to inhibit the ability of TRF1 to induce supercoiling, as deletion of the acidic domain (TRF1ΔA) enhances the ability of TRF1 to promote supercoiling. Likewise, replacement of the basic domain of TRF2 with the acidic domain of TRF1 (TRF2hAΔB) weakens the ability of TRF2 to promote supercoiling.[24] The ability of TRF-derived proteins to modulate Rad51-mediated telomeric D-loop formation is negatively correlated with their own ability to directly promote telomeric D-loop formation (see Table 1). Likewise, the ability of TRF-derived proteins to directly promote telomeric D-loop formation correlates with their ability to induce positive supercoiling within telomeric dsDNA.[12,24] This supercoiling may inhibit Rad51-mediated D-loop formation. The fact that TRF1 does not inhibit Rad51-mediated D-loop formation is consistent with this hypothesis.

Previous characterizations of TRF1 and TRF2 binding properties have also revealed that whereas TRF2 binds to telomeric dsDNA as a large oligomeric structure, TRF1 does not oligomerize to the same degree.[24,26] The dimerization domain and linker domain of TRF2 have both been implicated in its oligomerization.[24−26,29] The N-terminal domains of TRF1 and TRF2 may also affect oligomerization. The presence of the acidic domain on either TRF1 or TRF2hAΔB or the absence of the basic domain from TRF2ΔB reduces the average size of the complexes these proteins form upon binding to telomeric dsDNA.[24] The lower-order binding characteristics of TRF1 combined with its inability to induce supercoiling may permit it to promote Rad51-mediated telomeric D-loop formation via an unknown mechanism.

Although TRF1 and TRF2 are found at telomeres throughout the cell cycle and TRF1 promotes efficient telomeric replication *in vivo*,[7] TRF1 and TRF2 inhibit DNA replication *in vitro*.[6] However, TRF1 binding and TRF2 binding are inhibited by post-translational modifications (PTMs) *in vivo*, some of which are conferred by replication complex-associated proteins.[30−36] Interestingly, PTMs that reduce the level of TRF1 binding are inhibited *in vivo* by another shelterin protein, TIN2,[30] and by FANCD2, a component of the Fanconi anemia pathway.[33] Comparable PTMs of TRF2 are not likewise inhibited. As TRF1 promotes and TRF2 inhibits Rad51-mediated telomeric D-loop formation, depletion of TRF2 but not TRF1 from DNA near the replication fork may facilitate HR-mediated fork restart within the telomeres (Figure 6A). However, these TRF2 PTMs likely disrupt TRF2 dimerization, which may abrogate Myb domain binding but permit basic domain binding. Replication fork restart may also be facilitated by basic domain-mediated recruitment of TRF2 to regressed forks, where it can both protect the nascent Holliday junction (HJ) from HJ resolvases[22,28] and recruit RecQ helicases that can promote fork migration.[37−39] Likewise, the presence of TRF2 on telomeric dsDNA away from the fork may prevent HR-mediated strand invasion reactions and protect the telomeres from aberrant repair (Figure 6B).

dx.doi.org/10.1021/bi5006249 | *Biochemistry* 2014, 53, 5485−5495

We observed that TRF2ΔM promoted Rad51-mediated nontelomeric D-loop formation, despite the ability of TRF2 to inhibit Rad51-mediated telomeric D-loop formation. This suggests that TRF2 possesses domains that both positively and negatively modulate Rad51-mediated D-loop formation. While TRF2ΔM did not promote Rad51-mediated telomeric D-loop formation across the entire Rad51 concentration range tested, TRF2ΔM did significantly promote Rad51-mediated telomeric D-loop formation at the lowest Rad51 concentration tested (Figure 3A). We speculate that the high efficiency of telomeric D-loop formation in reactions with high Rad51 concentrations may mask the ability of TRF2ΔM to promote this activity. TRF2ΔM binds telomeric DNA with low affinity and specificity, likely via the basic domain, which has been shown to direct binding in a sequence-independent manner to unusual DNA structures.[5] Interestingly, the basic domain and TRF2ΔM can create or stabilize open dsDNA structures and promote DNA junction mobility similar to that required for D-loop formation.[24] These processes are similar to the activities of Rad54, a Rad51 accessory protein. Rad54 binds to dsDNA and promotes its unwinding *in vitro* and promotes Rad51-mediated D-loop formation.[13] In the absence of Myb domain-directed binding, the basic domain of TRF2 may promote this process in a similar manner.

The role of TRF2 in the HR pathway is not well-understood. TRF2 is recruited to dsDNA breaks (DSBs) in a basic domain-dependent but not Myb domain-dependent manner[40] and can occur in an ATM deficient background. TRF2 is phosphorylated by ATM[41] in response to DNA damage,[42] and mutations that disrupt TRF2 phosphorylation inhibit DNA repair.[43] While it has been suggested that this DNA repair defect may be due to impaired nonhomologous end joining (NHEJ),[42] the defect may also be due to impaired HR. Overexpression of TRF2 and TRF2ΔM promotes HR *in vivo*.[11] Likewise, knockdown of TRF2 inhibits HR but not NHEJ *in vivo*.[11] Our finding that TRF2ΔM can promote Rad51-mediated D-loop formation suggests a novel mechanism by which TRF2 can promote HR (Figure 6C). Upon induction of a DSB, TRF2 may undergo basic domain-mediated recruitment to the site of damage. TRF2 may then help recruit proteins such as the Mre11/Rad50/Nbs1 (MRN) complex,[44] which promotes end resection in preparation for HR. Following end resection, Rad51 binding, and a homology search, the basic domain of TRF2 may facilitate Rad51-mediated D-loop formation by promoting the opening of the template dsDNA in a manner similar to that of Rad54,[13] or by promoting DNA joint migration.[22]

This model of functional interactions among TRF1, TRF2, and Rad51 provides insight into both telomere biology and the HR pathway. Previous characterizations suggested that TRF2 and Rad51 cooperate to promote telomeric D-loop and possibly t-loop formation *in vivo*, despite apparent incompatibilities in the *in vitro* activities of these proteins. Our finding that TRF2 inhibits Rad51-mediated telomeric D-loop formation suggests that Rad51 does not contribute to t-loop formation or that this inhibition must be alleviated by additional factors *in vivo*. While it has previously been reported that TRF1 is required for efficient telomere replication *in vivo*, this requirement seems at odds with other reports that TRF1 can inhibit telomere replication *in vitro*. Our finding that TRF1 promotes Rad51-mediated telomeric D-loop formation suggests that TRF1 may facilitate telomere replication by promoting HR-mediated replication fork restart. Finally, our observation that TRF2ΔM can promote Rad51-mediated nontelomeric D-loop formation may explain how TRF2 can promote HR *in vivo*.

■ ASSOCIATED CONTENT

Supporting Information

Figures S1−S5. This material is available free of charge via the Internet at http://pubs.acs.org.

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Funding

This work was supported by National Institutes of Health Grants GM31819, ES3773, and GM007092.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank Dr. Richard Fishel for providing the Rad51 expression vector and Dr. Ravindra Amunugama for providing technical advice about optimizing our fluorescent D-loop assay. We thank Dr. Eric Gilson for providing a TRF2 expression vector that was modified for use in this project. We appreciate Dr. Sezgin Özgür's advice and assistance with protein purifications.

■ ABBREVIATIONS

AUC, area under the curve; ATM, ataxia telangiectasia mutated; BSA, bovine serum albumin; cDNA, coding DNA; Cy3, cyanine 3; D-loop, displacement loop; DTT, dithiothreitol; dsDNA, double-stranded DNA; EMSA, electrophoretic mobility shift assay; FANCD2, Fanconia anemia group D2 protein; HJ, Holliday junction; HR, homologous recombination/repair; kbp, kilobase pair; MRN, Mre11/Rad50/Nbs1 complex; NHEJ, nonhomologous end joining; nt, nucleotide; PCR, polymerase chain reaction; PTM, post-translational modification; ssDNA, single-stranded DNA; ss−dsDNA, single-stranded−double-stranded DNA; SDS, sodium dodecyl sulfate; t-loop, telomere loop; TRF1, telomere repeat binding factor 1; TRF2, telomere repeat binding factor 2; TIN2, TRF1 interacting nuclear protein 2.

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